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Review

Specific interactions of the adenovirus proteinase with the viral DNA, an 11-amino-acid viral peptide, and the cellular protein actin

W. F. Mangel[a,*], M. L. Baniecki[b] and W. J. McGrath[a]

[a] Biology Department, Brookhaven National Laboratory, 50 Bell Avenue, Upton, New York 11973 (USA), Fax +1 631 344 3407, e-mail: mangel@bnl.gov
[b] Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, New York 11974 (USA)

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Abstract. The adenovirus proteinase (AVP) is synthesized in an inactive form that requires cofactors for activation. The interaction of AVP with two viral cofactors and with a cellular cofactor, actin, is characterized by quantitative analyses. The results are consistent with a specific model for the regulation of AVP. Late in adenovirus infection, inside nascent virions, AVP becomes partially activated by binding to the viral DNA, allowing it to cleave out an 11-amino-acid viral peptide, pVIc, that binds to AVP and fully activates it. Then, about 70 AVP-pVIc complexes move along the viral DNA, via one-dimensional diffusion, cleaving virion precursor proteins 3200 times to render a virus particle infectious. Late in adenovirus infection, in the cytoplasm, the cytoskeleton is destroyed. The amino acid sequence of the C terminus of actin is homologous to that of pVIc, and actin, like pVIc, can act as a cofactor for AVP in the cleavage of cytokeratin 18 and of actin itself. Thus, AVP may also play a role in cell lysis.

Key words. Actin; cofactor; cysteine proteinase; cytokeratin 18; precursor protein.

Introduction

Human adenovirus encodes a proteinase (AVP) whose activity is essential for the synthesis of infectious virus [1]. Properties of a temperature-sensitive mutant (ts-1) indicate that one of the functions of the proteinase, after virion assembly, is to cleave six major virion precursor proteins to the mature counterparts found in wild-type virus [2, 3]. There are ~70 molecules of AVP per virion [4], and they must cleave at 3200 sites to render a virus particle infectious. Another function of AVP late in adenovirus infection is to cleave cytokeratin 18, leading to the destruction of the cytokeratin network [5]. The ts-1 mutation mapped to the L3 23K gene [1, 6]. This gene was cloned and expressed in *Escherichia coli* [7–9] or baculovirus-infected insect cells [10] and the resultant 204-amino-acid protein purified.

AVP is unusual in that it requires cofactors for maximal activity. Proteinase activity can be observed in wild-type virus but not in ts-1 virus [7, 10]. Unexpectedly, no proteinase activity could be observed with purified, recombinant AVP. However, purified, recombinant AVP in vitro complemented the mutation in ts-1 virions, restoring proteinase activity when mixed together. This implied that cofactors may be required. One viral cofactor, pVIc, is the 11-amino-acid peptide (GVQSLKRRRCF) derived from the C terminus of virion precursor protein pVI. Preceding the sequence for pVIc is the AVP consensus cleav-

* Corresponding author.

age sequence IVGL-G in which cleavage occurs between L and G and G becomes the N-terminal amino acid residue of pVIc. Thus, AVP can cleave pVI to liberate its own cofactor. The other viral cofactor is the viral DNA [7, 11]. A third cofactor is the abundant cytoplasmic protein actin [12]. AVP is relatively inactive; the cofactors bind to AVP and activate the enzyme [13].

The crystal structure of the AVP-pVIc complex is known at a resolution of 2.6 Å [14], and it revealed that AVP is not structurally homologous to any protein structure in the databases (fig. 1). However, AVP shares some common secondary-structural elements with papain [13]. When the common secondary-structure elements are aligned and the amino acids of the active-site region of papain [15] are compared to amino acids in the same positions in AVP, AVP is clearly seen to be a new type of cysteine proteinase. In positions identical to Cys25, His159 and Asn175 of papain are Cys122, His54 and Glu71 of AVP, respectively. Even Gln19 of papain, presumed to participate in the formation of the oxyanion hole [16], aligns with Gln115 of AVP. The main-chain nitrogen atoms of the two active-site cysteine residues also match; in papain this atom is proposed to join with Gln19 to form the oxyanion hole [17]. This remarkable juxtaposition of catalytic elements strongly suggested that AVP employs the same catalytic mechanism as papain [18] and that AVP is an example of convergent evolution [13].

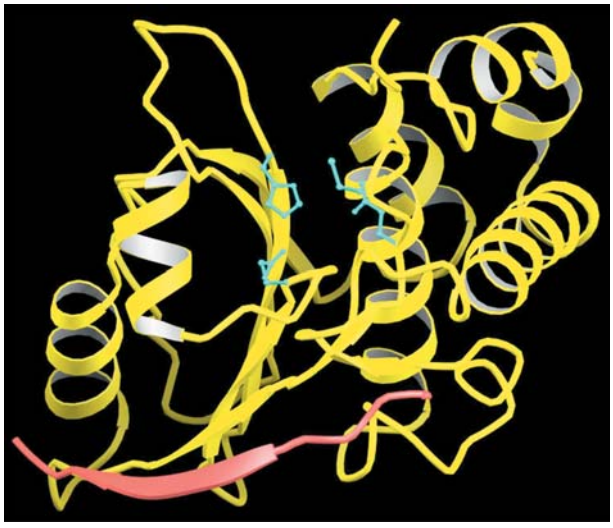

Figure 1. The active site and secondary structure of the AVP-pVIc complex [14]. The pVIc peptide is colored red. Side chains, in turquoise, are shown only for the active-site residues Cys122, His54, Glu71 and Gln115.

Binding of the viral cofactors to AVP alters the macroscopic kinetic constants for the hydrolysis of the fluorogenic substrate $(Leu\text{-}Arg\text{-}Gly\text{-}Gly\text{-}NH)_2$-rhodamine [7, 19]. In the absence of any cofactor, the K_m is 94.8 µM [11] and k_{cat} is 0.002 s^{-1} (table 1). In the presence of a saturating amount of Ad2 DNA, the K_m decreases 10-fold and the k_{cat} increases 11-fold; in the presence of a saturating amount of pVIc, the K_m decreases 10-fold and the k_{cat} increases 118-fold. With saturating amounts of both cofactors, the k_{cat}/K_m ratio increases 34,000-fold compared to that for AVP alone. Here, the specific interactions of AVP with the two viral cofactors pVIc and DNA as well as with actin are described.

Interaction of AVP with its cofactor pVIc, the 11-amino-acid viral peptide

The penultimate amino acid residue in pVIc, Cys10′, is important in the binding of pVIc to AVP. In the AVP-pVIc crystal structure (fig. 1), the conserved amino acid Cys10′ of pVIc forms a disulfide bond with the conserved amino acid Cys104 of AVP [14]. That bond can form in two ways. pVIc can form a homodimer via disulfide bond formation with a second-order rate constant of 0.12 M^{-1} s^{-1} [20], and half of the homodimer can covalently bind to AVP via thiol-disulfide exchange [10, 20]. Alternatively, monomeric pVIc could form a disulfide bond with AVP via oxidation [20]. The K_d for the reversible binding of pVIc to AVP is 4.4 µM. The K_d for the binding of the pVIc mutant C10′A is at least 100-fold higher; surprisingly, the K_d for the pVIc mutant decreased at least 60-fold, to 6.93 µM, in the presence of 12-mer single-stranded DNA (ssDNA). Furthermore, once the pVIc mutant C10′A is bound to an AVP-DNA complex, the macroscopic kinetic constants for substrate hydrolysis are the same as those exhibited by wild-type pVIc.

The cysteine in pVIc is important in the binding of pVIc to AVP, but formation of a disulfide bond between pVIc

Table 1. The effect of cofactors on the macroscopic kinetic constants for substrate hydrolysis by AVP [11].

	K_m (µM)	$k_{cat} \times 10^3$ (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	relative k_{cat}/K_m*
AVP	94.8 ± 7.0	2.3 ± 0.1	24.3	1
AVP-DNA†	9.2 ± 1.4	24.8 ± 1.9	2700	110
AVP-pVIc	9.9 ± 1.3	271 ± 32.2	27,400	1130
AVP-pVIc-DNA†	3.4 ± 1.4	2780 ± 322	828,000	34,100

* (k_{cat}/K_m) divided by the (k_{cat}/K_m) for AVP.
† DNA is adenovirus DNA.

and AVP is not required for maximal stimulation of enzyme activity by pVIc. The kinetics of formation of a disulfide bond between AVP and pVIc and the effect of 12-mer ssDNA on the formation of that bond are shown in figure 2. The pVIc dimer forms a disulfide bond to AVP with a half-time of 25 s. The half-time for formation of a disulfide bond with monomeric pVIc is much longer, 1740 s. This is expected as thiol-disulfide exchange is much faster than oxidation. The presence of DNA has a slight effect on the rate of formation of a disulfide bond between monomeric pVIc and AVP; the half-time decreased 26%, to 1288 s. The time to attain the maximal rate of substrate hydrolysis upon addition of pVIc to AVP, in the presence and absence of DNA, is shown in figure 3. Upon addition of monomeric pVIc, there is a lag of ~180 s before the maximal rate of substrate hydrolysis is achieved. In the presence of DNA, the lag is much shorter, with the maximal rate occurring within 7 s of mixing. Since formation of the disulfide bond takes longer than achievement of the maximal rate of substrate hydrolysis, formation of a disulfide bond is therefore not necessary for maximal activation of the enzyme by pVIc. Although formation of a disulfide bond between Cys104 of AVP and Cys10′ of pVIc is not required for maximal stimulation of AVP activity, it may be required to keep AVP permanently activated in the virion. Perhaps the role of pVIc in the activation of AVP is to form a strap between the two domains of AVP that optimally aligns the Cys-His dyad to a geometry which favors the maintenance of the ion pair. Given the relatively low concentration of pVIc in the virion, reversible binding of pVIc to AVP might not allow the enzyme to be sufficiently activated so that it can cleave all the virion precursor proteins. One way to ensure sufficient activation is the formation of a disulfide bond. In vivo in the virus particle, pVIc is covalently linked to AVP [21].

The reversible interaction of AVP and AVP-DNA complexes with pVIc has been quantitatively characterized [22]. The K_d for the binding of pVIc to AVP is 4.4 µM. The binding of AVP to 12-mer ssDNA decreases the K_d for the binding of pVIc to AVP to 0.09 µM. Alanine-scanning mutagenesis, in which each amino acid of pVIc is individually replaced with an alanine residue, has been used to determine the contribution of individual pVIc side chains in the binding and stimulation of AVP (table 2). Two amino acid residues, Gly1′ and Phe11′, are the major determinants in the binding of pVIc to AVP, while Val2′ and Phe11′ are the major determinants in stimulating enzyme activity. Most of the free energy of binding of pVIc to AVP is contributed by Gly1′ and the side chain of Phe11′. The $\Delta\Delta G^*_T$ upon substitution of an alanine for Gly1′ is 1.57 kcal/mol, and for substitution of an alanine for Phe11′, the $\Delta\Delta G^*_T$ is 1.15 kcal/mol. Both residues are largely sequestered from solvent in the complex, with only 20% of the surface area of Gly1′ accessible and 9% of the surface area of the Phe11′ side chain accessible. The solvent occlusion of these hot spots in pVIc is consistent with studies of protein-protein interfaces, showing that solvent exclusion is a necessary condition for tight

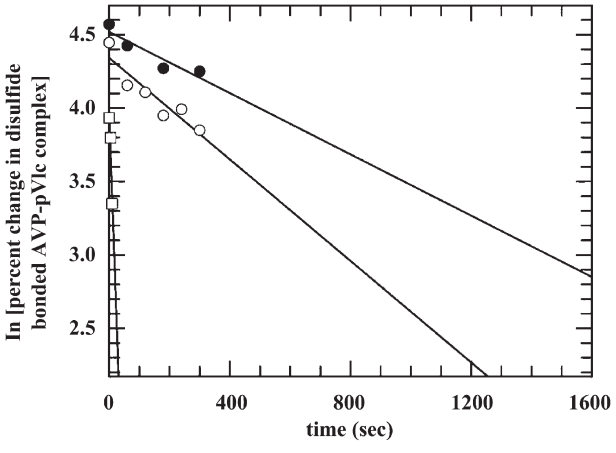

Figure 2. Kinetics of formation of a disulfide bond between AVP and pVIc in the absence or presence of 12-mer ssDNA [20]. AVP (12.5 µM) was preincubated at 37 °C for the indicated times in the presence of 20 µM monomeric pVIc (●), 0.5 µM monomeric pVIc and 1 µM 12-mer ssDNA (○) or 20 µM dimeric pVIc (□). Aliquots of these reactions were removed, diluted 500-fold to a concentration 100-fold lower than the K_d for the reversible binding of monomeric pVIc to AVP, and assayed for enzyme activity. For the control experiment, aliquots were removed from the initial reactions, diluted 500-fold but into the same concentrations of pVIc, or pVIc and DNA, or dimeric pVIc, and assayed for enzyme activity. The rates of substrate hydrolysis were divided by the rates of substrate hydrolysis from the control reactions. This ratio was multiplied by 100; the resultant number was subtracted from 100, and the natural logarithm of that number was plotted versus time. The slopes of the lines are 0.0017 (●), 0.001 (○) and 0.058 (□) s^{-1}.

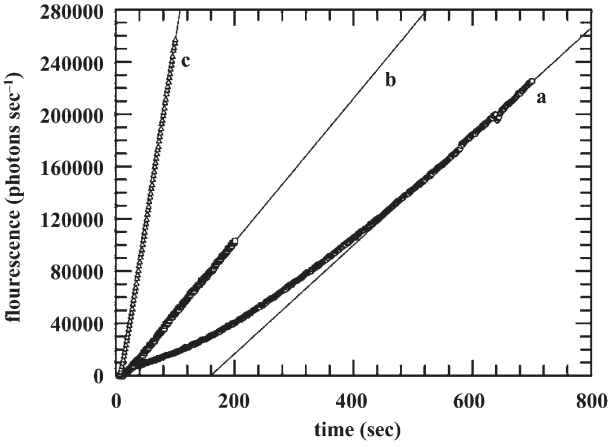

Figure 3. Time to attain the maximal rate of substrate hydrolysis upon addition of pVIc to AVP, in the presence or absence of DNA [20]. Monomeric pVIc (a), dimeric pVIc (b) or monomeric pVIc in assays containing 1 µM 12-mer ssDNA (c) was added to cuvettes containing 25 nM AVP and saturating concentrations of fluorogenic substrate, and the increase in fluorescence was monitored as a function of time.

Table 2. Alanine-scanning mutagenesis of pVIc – characterization of the interaction of the mutants with AVP in the absence or presence of DNA [20, 22].

Peptide		K_d (μM)	K_m (μM)	k_{cat} (s^{-1})
GVQSLKRRRCF	DNA (–)	4.43 ± 0.46	2.10 ± 0.76	1.08
	(+)	0.09 ± 0.01	3.83 ± 0.52	2.9
AVQSLKRRRCF	DNA (–)	56.09 ± 6.67	2.94 ± 1.40	1.15
	(+)	0.08 ± 0.01	1.69 ± 0.66	0.75
G**A**QSLKRRRCF	DNA (–)	5.59 ± 0.26	1.79 ± 0.63	0.16
	(+)	0.07 ± 0.02	2.87 ± 0.75	0.89
GV**A**SLKRRRCF	DNA (–)	0.04 ± 0.02	1.86 ± 0.74	0.41
	(+)	0.13 ± 0.04	7.01 ± 1.63	6.79
GVQ**A**LKRRRC	DNA (–)	4.19 ± 1.97	3.05 ± 0.91	0.52
	(+)	0.10 ± 0.03	1.23 ± 0.45	2.60
GVQS**A**KRRRCF	DNA (–)	0.53 ± 0.09	3.59 ± 0.57	0.61
	(+)	0.03 ± 0.01	3.68 ± 0.00	1.23
GVQSL**A**RRRCF	DNA (–)	5.86 ± 1.17	3.56 ± 0.95	0.50
	(+)	0.42 ± 0.09	1.83 ± 0.76	4.20
GVQSLK**A**RRCF	DNA (–)	0.28 ± 0.05	3.90 ± 0.92	0.50
	(+)	0.10 ± 0.04	1.27 ± 0.39	1.13
GVQSLKR**A**RCF	DNA (–)	3.17 ± 0.01	3.46 ± 1.60	0.84
	(+)	0.21 ± 0.02	4.53 ± 1.85	6.10
GVQSLKRR**A**CF	DNA (–)	0.80 ± 0.23	3.59 ± 1.30	0.56
	(+)	0.14 ± 0.06	2.39 ± 0.44	4.98
GVQSLKRRR**A**F	DNA (–)	>440	N.D.	N.D.
	(+)	6.93 ± 1.1	8.06 ± 2.14	0.43
GVQSLKRRRC**A**	DNA (–)	28.40 ± 4.05	1.21 ± 0.38	0.21
	(+)	0.24 ± 0.13	1.81 ± 0.54	0.73

Assays were performed in the absence and presence of 1 μM 12-mer ssDNA. In the presence of 12-mer DNA, the AVP concentration was 10 nM, and in the absence of 12-mer DNA, the AVP concentration was 20 nM. Binding and kinetic constants were calculated as described elsewhere [22]. N.D., not determined.

binding [23]. Binding of AVP to DNA greatly suppresses the effects of the alanine substitutions on the binding of mutant pVIcs to AVP. For example, the K_d for the binding of the pVIc mutant G1′A to AVP is 56 μM, and this decreases to 0.08 μM in the presence of DNA, the same as the K_d for the binding of wild-type pVIc to AVP in the presence of DNA.

Interaction of AVP with its cofactor the viral DNA

The interaction of AVP with Ad2 DNA as a cofactor is not dependent upon a specific nucleic acid sequence [7]. Various polymers were substituted for Ad2 DNA and assayed for cofactor activity. Not only did T7 DNA substitute for Ad2 DNA, but ssDNAs, circular single- and double-stranded DNAs, transfer RNAs and even polyglutamic acid but not polylysine also stimulated enzyme activity. The data are consistent with the conclusion that this cofactor requirement is for a polymer with high negative-charge density. Monomeric units of polymers with high negative-charge density, e.g. the four deoxyribonucleoside monophosphates or glutamic acid, did not substitute for Ad2 DNA or polyglutamic acid. The major polyanion with a high negative-charge density in the virus particle is, of course, the viral DNA.

That the viral DNA functions as a cofactor for proteinase activity is disputed [10, 24, 25]. We showed that the viral DNA is a cofactor in the Ad2 virion, because proteinase activity is lost upon treatment with DNase and restored upon addition of Ad2 DNA [7]. Second, we showed processing of virion precursor proteins in disrupted H2ts-1 virus upon incubation with AVP; however, no processing occurred if the disrupted virions were pretreated with DNase before the addition of AVP [26].

The interaction of AVP and of AVP-pVIc complexes with DNAs has been quantitatively characterized [11]. The K_d for the binding of AVP to 12-mer dsDNA is 63 nM, and for AVP-pVIc, it is 2.9 nM (table 3). The stoichiometry of binding is proportional to the length of the DNA (table 4), as predicted from the observations that the binding of AVP to DNA does not require a specific DNA sequence [7]. Three molecules of AVP-pVIc bind to 18-mer dsDNA and six molecules to 36-mer dsDNA [11]. The electrostatic component of binding of AVP-pVIc to 12-mer dsDNA originates from the formation of ion pairs between positively charged groups on AVP-pVIc and negatively charged phosphate groups on DNA. Two ion pairs are involved. The nonelectrostatic free energy of binding is –4.6 kcal. Thus, a substantial component of the binding free energy under physiological conditions results from nonspecific interactions between AVP-pVIc and base or

Table 3. Equilibrium dissociation constants for the binding of AVP, pVIc and AVP-pVIc complex to single or double-stranded 12-mer DNA determined by enzyme activity or fluorescence anisotropy [11].

Protein	12 mer	K_d (nM) (by activity)	Kd (nM) (by anisotropy)
AVP	dsDNA	–	63.08 ± 5.79
	ssDNA	–	109.17 ± 15.60
pVIc	dsDNA	–	693.0 ± 84.47*
	ssDNA	–	190.4 ± 34.37*
AVP-pVIc	dsDNA	2.92 ± 1.04	4.56 ± 2.32
	ssDNA	18.04 ± 5.08	18.40 ± 2.80

* $K_{d\ (apparent)}$.

sugar residues on the DNA. This indicates the dominant factor driving the nonspecific binding interaction between AVP-pVIc and DNA is the entropic contribution from the release of counterions. Perhaps the most convincing evidence that DNA is a cofactor for AVP comes from the experiment in which increasing amounts of 12-mer dsDNA or 5′ fluorescein-labeled 12-mer double-stranded DNA (dsDNA) are added to a constant amount of AVP-pVIc and, respectively, either enzyme activity or the change in anisotropy measured. The data points from both types of assay are superimposable, indicating that binding of DNA to AVP-pVIc is coincident with stimulation of AVP-pVIc activity by DNA (fig. 4).

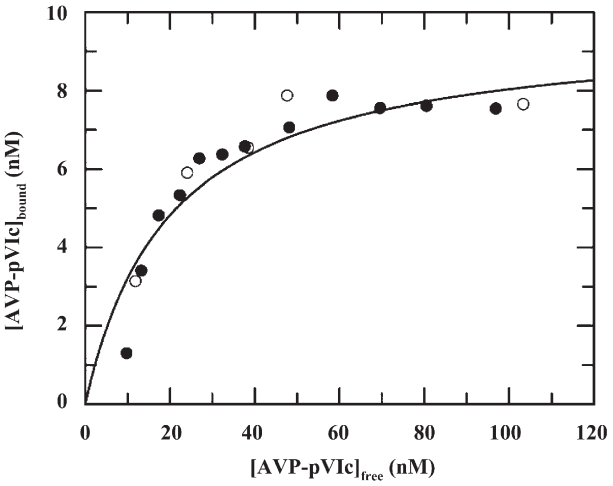

Figure 4. Illustration of the coincidence of AVP-pVIc complex binding to DNA and stimulation of enzyme activity by DNA [11]. Increasing concentrations of AVP-pVIc were added to 10 nM 5′ fluorescein-labeled 12-mer ssDNA at 25 °C. Two minutes after each addition, the change in anisotropy was measured (●). Increasing concentrations of AVP-pVIc were added to 10 nM 12-mer ssDNA. After 5 min at 25 °C, substrate was added, and the increase in fluorescence with time was measured (○). The concentrations of bound AVP-pVIc ($[AVP\text{-}pVIc]_{bound}$) and free AVP-pVIc ($[AVP\text{-}pVIc]_{free}$) were determined as described in McGrath et al. [11].

Interaction of AVP with a cellular cofactor, actin

Throughout an adenovirus infection, the actin, cytokeratin, tubulin and vimentin networks that make up the cell cytoskeleton undergo dramatic changes [27]. Chen et al. [5] have shown that late in an adenovirus infection, cytokeratin 18 is cleaved at two contiguous AVP consensus cleavage sequences, leading to the destruction of the cytokeratin network. This observation raised a conundrum. Cleavage of cytokeratin 18 by AVP takes place in the cytoplasm, but AVP is synthesized in an inactive form and is activated within immature virions in the nucleus by the two viral cofactors.

Does AVP need a cofactor to cleave cytokeratin 18? To determine if AVP needs a viral component to act as a cofactor in the cleavage of cytokeratin18, an AVP-green fluorescent protein (GFP) chimeric gene was transfected into HeLa cells [12]. Soon after transfection, AVP was found in the cytoplasm where it colocalized with cytokeratin 18; later, the cytokeratin network was destroyed. Thus, AVP can be active in the cytoplasm in the absence of other viral components. Does AVP need a cofactor to cleave cytokeratin 18? Apparently it does. Incubation of a cytokeratin-18-enriched HeLa cell fraction with AVP resulted in no cleavage of cytokeratin 18. However, under the same conditions but in the presence of pVIc, cytokeratin 18 is cleaved.

Actin was considered a potential cytoplasmic cofactor for AVP, because the C-terminal amino acid sequence of actin is highly homologous to the amino acid sequence of

Table 4. Equilibrium dissociation constants, K_d, and stoichiometries of binding of AVP-pVIc to different DNAs determined by enzyme activity [11].

		12-mer	18-mer	36-mer	Ad2
K_d (nM)*	ds	2.92 ± 1.04	5.97 ± 1.09[a]	4.65 ± 2.16[a]	–
	ss	18.04 ± 5.08	8.88 ± 1.93[a]	2.13 ± 0.41[a]	
Stoichiometry[b]	ds	1:1	3:1	6:1	3027:1
	ss	1:1	3:1	6:1	

* Concentration in units of molecules of DNA.

[a] $K_{d\ (apparent)}$.

[b] Ratio is the number of AVP-pVIc molecules binding per DNA molecule.

pVIc (fig. 5A, B). Of the last eight amino acid residues of actin, four are identical and three are homologous to the last eight amino acid residues in pVIc. Comparison of the ten C-terminal amino acid residues in the α-, β- and γ-actin isomers reveals that these residues are strictly conserved. The penultimate amino acid in actin is Cys374 and the penultimate amino acid in pVIc is Cys10′. For actin, in particular for its C terminus to be a cofactor for AVP, the C terminus must be accessible to interact with AVP. Inspection of the crystal structure of actin [28] or an actin-profilin complex [29] shows that the C-terminus of actin is on the surface and could, therefore, be accessible to interact with AVP.

The interaction between actin and AVP is beginning to be characterized [12]. When increasing concentrations of monomeric actin (G-actin) are incubated with AVP, the rate of substrate hydrolysis increases in proportion to the actin concentration, indicating that actin is indeed a cofactor (fig. 6). As predicted from the sequence homology, AVP binds to the C terminus of actin, because the fluorescence from actin labeled with PRODAN at Cys374 is quenched upon incubation with AVP. Measurement of the equilibrium dissociation constant for the binding of actin to AVP shows that the binding is very tight; the K_d is 4 nM. Will actin act as a cofactor in the cleavage of cytokeratin 18? Yes: AVP and actin were incubated with the cytokeratin-18-enriched HeLa cell fraction and cleavage was observed. Analysis of the amino acid sequence of β-actin reveals two AVP consensus cleavage sequences, one at the N terminus and one at the C terminus (fig. 5A). This raised the possibility that actin is not only a cofactor for AVP, but is also a substrate for AVP. Accordingly, actin and AVP were incubated together. Subsequent SDS-PAGE analysis indicated that actin can indeed be cleaved by AVP. AVP preferentially cleaved at the N terminus of actin yielding a 40-kDa fragment which was then cleaved at its C terminus to yield bands of 29 and 11 kDa. Thus, actin is indeed a substrate for AVP.

In virus-infected cells, cleavage of cytoskeletal proteins weakens the mechanical structure of the cell, and this may promote cell lysis and release of nascent virions [5]. AVP cleaves cytokeratin 18 within the N-terminal domain yielding a 41-kDa fragment that is incapable of participating in filament elongation. Such fragments significantly inhibit the elongation of cytokeratin filaments, even when the amount of cleaved cytokeratin comprises only 1% of the population. Inspection of the amino acid sequences of other cytoskeletal proteins reveals AVP consensus cleavage sequences in tubulin, vimentin and even actin itself. The latter observation raised the possibility that actin may be a cofactor for its own destruction, and this was shown to be the case. Degradation of cytoskeletal proteins by a virus-coded proteinase during lytic infections is not unusual. The rhinovirus 2A proteinase cleaves cytokeratin 8 [30], and other virus-coded proteinases cleave actin [31, 32] and vimentin [33]. What is currently unique about AVP is that it uses actin as a cofactor.

A. β-actin Sequence[1]

MDDDIAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDE
AQSKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKA
NREKMTQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVMDSGDGVTHTVPIYEGYAL
PHAILRLDLAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMA
TAASSSSLEKSYELPDGQVITIGNERFRCPEALFQPSFLGMESCGIHETTFNSIMKC
DVDIRKDLYANTVLSGGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIG
GSILASLSTFQQMWISKQEYDESGPSIVHRKCF

[1]AVP consensus cleavage sequences are underlined.

B. Comparison of pVIc sequence to sequences at the C-termini of various actins[2,3]

pVIc	GVQ**S**LKR**R**R**CF**
α-actin C-terminus	AGP**S**IVH**R**K**CF**
β-actin C-terminus	SGP**S**IVH**R**K**CF**
γ-actin C-terminus	SGP**S**IVH**R**K**CF**

[2]identity in amino acid residues, in **bold**
[3]homology in amino acid residues, underlined

Figure 5. The C terminus of actin is homologous to viral cofactor pVIc and actin can function as a cofactor in stimulating AVP activity [12]. The amino acid sequence of β-actin (*A*) and a comparison of the amino acid sequences of pVIc to the C termini of actin isomers (*B*) are shown. In (*A*), AVP consensus cleavage sites are underlined. In (*B*), amino acid residues in bold indicated identity in sequence; underlined amino acid residues indicate homology in sequence.

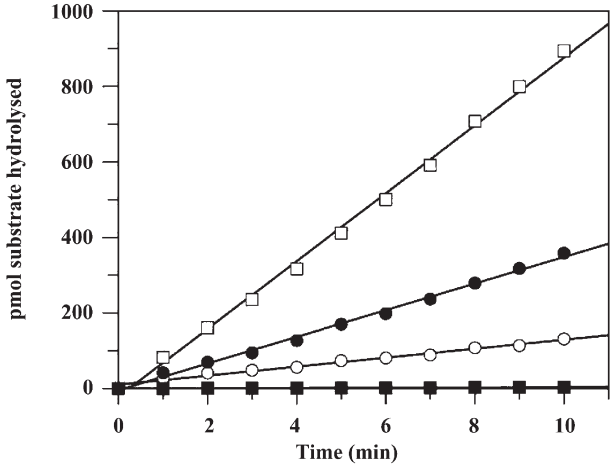

Figure 6. Illustration of the stimulation of AVP activity by actin [12]. Increasing concentrations of G-actin at 0 (■), 10 (○), 20 (●) and 50 (□) nM were incubated with 50 nM recombinant AVP for 5 min at 37 °C, after which 3 µM substrate was added, and the increase in fluorescence (pmol substrate hydrolyzed) measured as a function of time.

Model for the regulation of AVP in the virion by the two viral cofactors pVIc and DNA

The utilization of cofactors by AVP restricts its activity in both space and time. DNA binding activates AVP and localizes the enzyme to the DNA. This and other observations have led to a model (fig. 7) for the regulation of AVP by its viral cofactors: AVP is synthesized as a relatively inactive enzyme. The K_m for $(Leu\text{-}Arg\text{-}Gly\text{-}Gly\text{-}NH)_2$-rhodamine is 95 µM and the k_{cat} is 0.002 s^{-1}. If AVP were synthesized as an active enzyme, it would probably cleave virion precursor proteins before virion assembly, thereby preventing the formation of immature virus particles. Consistent with this hypothesis is the observation that if exogenous pVIc is added to cells along with Ad5 virus, the level of synthesis of infectious virus in those cells is severely diminished [22, 34, 35].

Quite possibly, AVP enters empty capsids bound to the viral DNA and remains bound during the maturation of the virus particle. AVP enters empty capsids bound to the viral DNA because the K_d for the binding of AVP to 12-mer dsDNA is quite low (63 nM). Inside the young virion, the viral DNA is positioned next to the C terminus of virion protein pVI. Protein VI is a DNA-binding protein [36]. AVP is partially activated by being bound to the viral DNA. Compared to the values with AVP alone, the K_m decreases 10-fold and the k_{cat} increases 11-fold. Thus, AVP-DNA complexes can cleave pVI at the proteinase consensus cleavage site preceding the amino acid sequence of pVIc. The liberated pVIc can then bind either to the viral DNA ($K_{d\,(apparent)}$ = 693 nM), to AVP molecules in solution (K_d = 4400 nM) or, most likely, to the AVP-DNA complex that liberated it (K_d = 90 nM) [22]. Once pVIc is bound to AVP, the penultimate cysteine in pVIc forms a disulfide bond with Cys104 of AVP [14, 20, 21]. AVP is now permanently activated. Compared to the kinetic constants with AVP alone, with pVIc-AVP-DNA, the K_m has decreased 28-fold and the k_{cat} has increased 1209-fold [11]. How can 70 fully activated proteinases bound to the viral DNA inside the virion [4] cleave precursor proteins 3200 times to render a virus particle infectious? For this to occur, either the enzymes or substrates must move inside young virions. Perhaps the proteinase moves along the viral DNA searching for processing sites on precursor proteins much like the *E. coli* RNA polymerase holoenzyme moves along DNA searching for a promoter. AVP-pVIc and RNA polymerase both exhibit an appreciable, non-sequence-specific affinity for DNA. The K_d values are 60 and 100 nM, in nucleotide base pairs, for AVP-pVIc and RNA polymerase [37], respectively.

RNA polymerase binds to DNA via a two-step mechanism. Initially, RNA polymerase binds to any place on DNA via free diffusion in three-dimensional space. Next, it dissociates from the DNA to a point where, though free to move, it is still near the original binding site. This enables it, with high probability and within a short period of time, to reassociate with the same or a nearby binding site. Once it locates a promoter, it exhibits an enormous

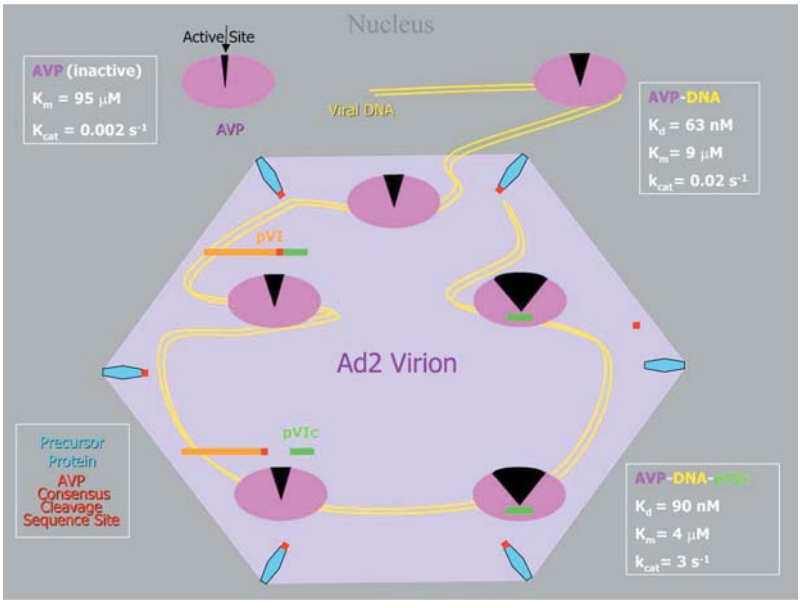

Figure 7. Model for the regulation of AVP by its viral cofactors. The diagram depicts AVP (inactive) in the nucleus. It binds to the viral DNA and enters the virus particle (hexagon) bound to the viral DNA. Partially activated by being bound to the viral DNA, AVP cleaves the precursor to protein VI, pVI, cutting out the 11-amino-acid peptide pVIc. pVIc then binds to the AVP-DNA complex. AVP is now fully activated and begins to use the viral DNA as a guide wire to locate viral precursor proteins. Seventy AVP molecules cleave virion precursor proteins 3200 times at AVP consensus cleavage sequences to render a virus particle infectious.

affinity for that sequence (K_d = 10 fM in nucleotide pairs) [37]. Because of 'nonspecific' binding, the search process for the promoter in the second step occurs in reduced dimensionality or volume. This is how RNA polymerase can reach a promoter at a rate faster than diffusion controlled – diffusion is occurring in one dimension.

In the case of AVP, perhaps the viral DNA serves as a scaffold next to which reside the 3200 processing sites that must be cleaved. The 70 AVP-pVIc complexes could then move along the viral DNA via one-dimensional diffusion using the DNA as a guide wire in cleaving precursor proteins. By using the viral DNA as a guide wire, AVP could quickly (via one-dimensional diffusion) and efficiently (by the alignment of the cleavage site near the DNA and by moving along the DNA) process the numerous virion precursor proteins. All of the six precursor proteins known to be processed by AVP are either bound to the viral DNA or adjacent to the viral DNA inside the virion – pIIIa, pVI, pVII, pVIII, pμ and pTP [38].

The future – AVP-like proteinases and their cofactors

When we started working on AVP, everything about it appeared to be unique. The sequence of the gene was unique, the regulation of enzyme activity by cofactors was unique, the fold of the proteinase was unique. Recently, enzymes have been discovered with similar characteristics. The hepatitis virus NS3 protease, which is a serine proteinase, utilizes a cofactor, the 54-residue peptide NS4A [39]. The cofactor, which can be mimicked by a 13-amino-acid peptide comprising residues 22–34 of NS4A, stimulates the k_{cat} for substrate hydrolysis [40]. The human rhinovirus 3C proteinase has been shown to bind directly to the viral RNA and to be important for the initiation of RNA replication [41]. During the virus lifecycle, 3C is also found fused to the 3D RNA polymerase. Both the autoprocessed form 3C and the fused form 3CD of the enzyme have proteolytic activity. A proteinase required for cell cycle progression in yeast, ubiquitin-like protease 1 (Ulp1), has sequence homology to AVP [42]. This enzyme cleaves SUMO-protein fusions and/or isopeptide-bond-linked SUMO-protein conjugates. A tandem pair of paralogous genes was identified in *Chlamydia* whose protein products are predicted to have a fold like that of AVP [43]. Both proteases are predicted to be integral membrane proteins. A virulence factor involved in signaling in *Yersinia pestis* is predicted to have secondary-structure homology to AVP [44]. The enzymes from yeast (Ulp1), *Chlamydia* and *Yersinia* appear to contain the same catalytic triad and oxyanion hole amino acids in the same relative position along the polypeptide chains as they are in AVP. Exploring whether these proteins are enzymes, whether they are active enzymes or require cofactors for activity will be part of an interesting future in this important and expanding field of biology.

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